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DETERMINATION OF L-FUCOSE IN GLYCOPROTEINS

II. An Automated Method for the Determination of L-Fucose in Serum Glycoprotein Hydrolysates

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DETERMINATION OF L-FUCOSE IN GLYCOPROTEINS

II. AN AUTOMATED METHOD FOR THE DETERMINATION OF L-FUCOSE IN SERUM GLYCOPROTEIN HYDROLYSATES

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ABSTRACT

An automated method is presented for the determination of L-fucose in serum glycoproteins. The procedure utilizes the cysteine-sulfuric acid reaction after mild acid hydrolysis of glycoprotein material. Details for flow manifold construction are presented together with data concerning the specificity and precision of the method.

I. INTRODUCTION

Studies of serum glycoprotein fucose levels in radiation therapy patients and of glycoprotein biosynthesis in irradiated animals produced a requirement for a specific and highly sensitive procedure for fucose analysis having a capability for large numbers of assays. The development of an automated method satisfying these requirements is the subject of this report.

The determination of the methylpentose L-fucose (6-deoxy-L-galactose) in glycoproteins is usually performed by Winzler's[6] modification of the cysteine-sulfuric acid reaction described by Dische and Shettles;[1] however, it has been demonstrated at this laboratory[5] and by other investigators[3,4] that nonfucose moieties of glycoproteins contribute spurious chromogens to the reaction. The fucose level obtained by Winzler's method, although a measure of methylpentose content, is not the true level when assaying samples containing large amounts of neutral hexoses (galactose and mannose).[5]

To circumvent this problem and thereby obtain a better estimation of the true fucose content of glycoproteins it is necessary to use one of three alternatives: first, to employ the specificity inherent in enzyme methods; second, to employ an internal standard;[1] or third, to reduce the effect (amount) of interfering nonfucose moieties in the cysteine-sulfuric acid reaction. With regard to the first, an enzyme assay employing L-fucose dehydrogenase isolated from pork liver has been proposed,[2] but the enzyme is not commercially available and its preparation is time consuming and beyond the capability of most clinical laboratories. The second alternative cannot be applied to the fucose analysis of glycoproteins[5] although it has been suggested

as the method of choice for biological samples.[1] Lastly, the third alternative could be achieved by introducing a preliminary acid treatment of the glycoprotein material to hydrolyze terminal fucose residues. The latter has been suggested by Gyorky and Houck[3] and forms the basis of the automated method proposed in this report.

II. MATERIALS

The reagents used on the flow manifold are those previously described by Dische and Shettles[1] and consist of 85 percent (v/v) sulfuric acid and 3 percent (w/v) aqueous cysteine hydrochloride. The concentration of working aqueous standards of L-fucose covers the range 5 to 20 μg/ml. Cysteine and working fucose standard solutions are prepared just prior to use.

III. PROCEDURE

Serum glycoproteins contained in 100 μl of serum were precipitated by the addition of 1 ml of 10 percent (w/v) trichloroacetic acid (TCA) and were centrifuged at 12,000 rpm for 10 minutes in a refrigerated Sorvall RC-2 centrifuge; the supernatant was decanted and discarded. Excess liquid was allowed to drain from the inverted centrifuge tubes for 10 minutes. The precipitate was then hydrolyzed in 1 ml of 0.6 N sulfuric acid for 75 minutes at 100°C. Optimum hydrolysis occurs in 0.6 N sulfuric acid[3] and is essentially complete in 75 minutes.[5]

After hydrolysis and cooling, solubilized proteins were reprecipitated with the addition of 1 ml of cold 10 percent TCA and recentrifuged as described above. The acid supernatant was used for analysis. TCA does not interfere with the fucose-cysteine-sulfuric acid color reaction.[3]

The flow diagram for the procedure using the AutoAnalyzer (Technicon Corporation, Tarrytown, New York) is shown in Figure 1. We have routinely operated the

system at a sampling rate of 40 per hour with a water wash between samples to obtain optimum resolution of sample peaks.

The hydrolysate is segmented with air, mixed with cold (5°C) sulfuric acid and heated to 95°C by passing the solution through a 40-foot glass coil submerged in an oil bath.

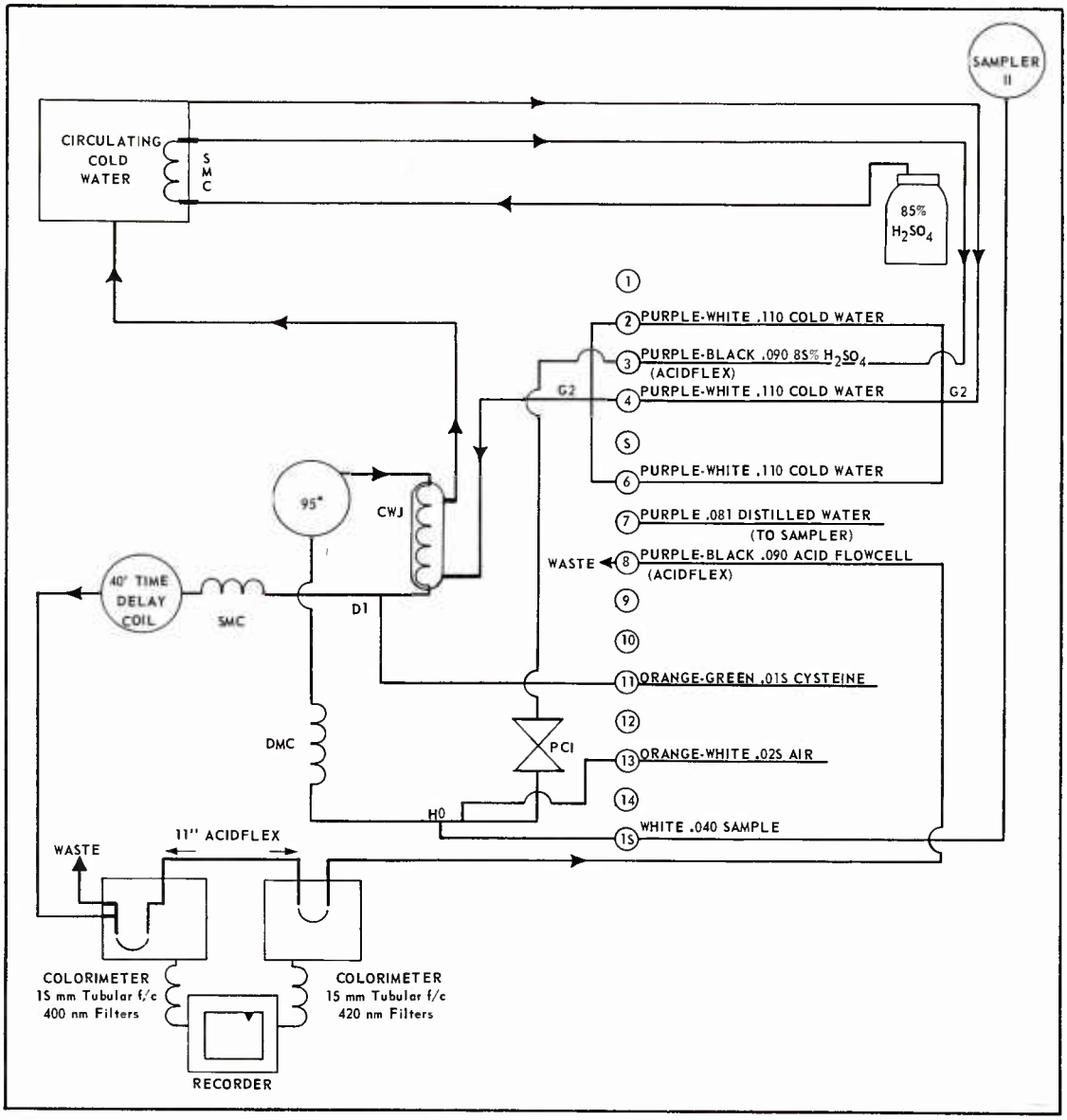

Figure 1. Diagram of the flow manifold for the automated determination of L-fucose. Glass fittings (Bulletin GF3, Technicon Corporation) and tube sizes are Technicon designations.

The cysteine reagent enters the stream after the acid solution has been cooled by a single pass through a cold (5°C) water-jacketed mixing coil. After a total delay of 37 minutes, from the time of sample aspiration, the yellow color produced in the reaction is measured at 400 nm and 420 nm in two 15-mm flowcell colorimeters connected in series. Correction for the chromogens produced by the small amount of nonfucose sugars present in the acid hydrolysate[3] is achieved by using the absorption at 420 nm as a measure of these chromogens[5] rather than 430 nm or 427 nm as reported by Dische and Shettles.[1] The time delay in the system was achieved by using both a proportioning pump modified to operate at 60 percent of its maximum speed and the 40-foot glass coil.

Results were calculated by comparing the increment in optical density, O. D. 400 nm - O. D. 420 nm, of the unknowns with that obtained with a series of standards and corrected by sample volume and dilution factors.

IV. RESULTS

Figure 2 shows the linear relationship between the increment in optical density (400 nm - 420 nm) and fucose concentration obtained with the automated technique. Figure 3 is an actual strip chart recording of a series of aqueous standards (5, 10, 20, and 40 μg/ml) followed by duplicate analyses of a sample obtained from a pool of human serum. The remaining six peaks were obtained by adding 20 μg of fucose contained in 1 ml of 0.6 N sulfuric acid to the human serum pool at the acid hydrolysis step described above. Although there is some carry-over from the highest standard to the first human pool sample, it had no effect on the increment, O. D. 400 nm - O. D. 420 nm, obtained for the two samples. The range in increment obtained with

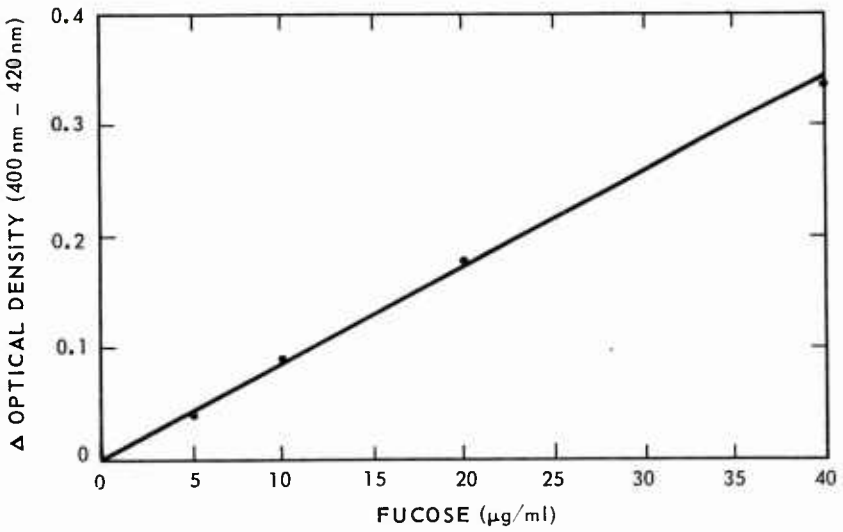

Figure 2. Positive increment in optical density, 400 nm - 420 nm, produced by various concentrations of L-fucose in an automated analytical system

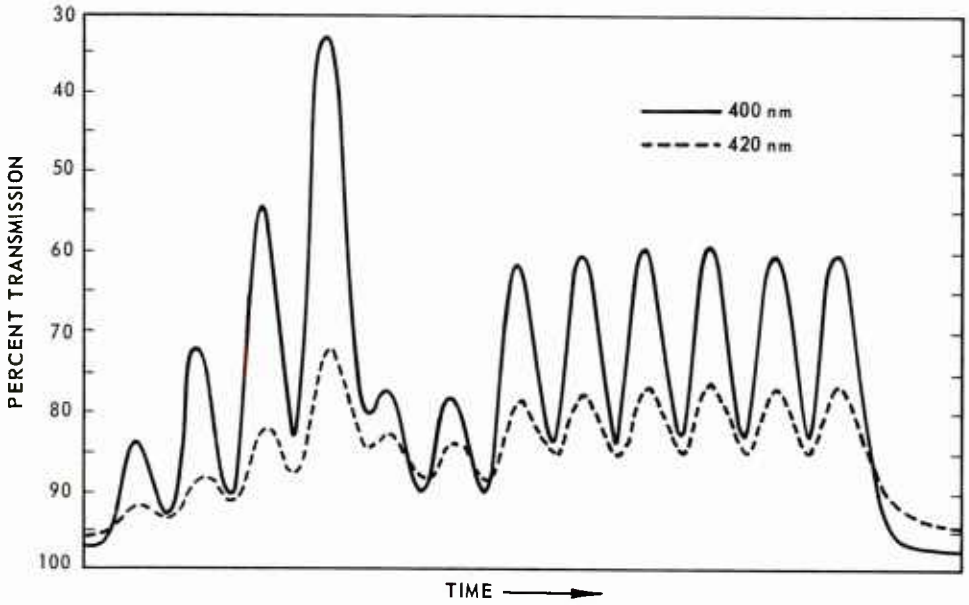

Figure 3. Actual strip chart recording of percent transmission obtained simultaneously at 400 nm and 420 nm with various samples. Identification of sample peaks is from left to right; peaks one through four represent 5, 10, 20, and 40 μg L-fucose per ml of distilled water, peaks five and six represent pooled human serum (PHS). The remaining six peaks are PHS with 20 μg of added fucose (see text).

the six human pool samples containing added fucose was 0.106 to 0.112 optical density units which is equivalent to a variation of 0.75 μg/ml.

Table I shows the results obtained when a human serum pool sample was assayed by the proposed automated method and by a manual enzyme method employing L-fucose dehydrogenase (FDH).[5] There is no significant difference between the mean values obtained by the two methods. The standard deviation for the automated method is one-half that of the enzyme method.

Table I. Fucose Content of a Human Serum Pool Obtained by Two Different Analytical Methods

Method*	Fucose (mg/100 ml)†
Automated (10)	7.2 ± 0.7
Manual, FDH‡ (10)	6.6 ± 1.4

* Number of replicate analyses is shown in parentheses

† Value shown is the mean ± the standard deviation

‡ L-fucose dehydrogenase methodology has been previously described[5]

V. DISCUSSION

Unlike the manual method originally proposed by Dische and Shettles,[1] the automated method does not require that optical density readings be obtained with and without cysteine. This is attributable to the elimination of many protein-bound organic substances from the reaction by the hydrolysis procedure used in the preliminary treatment of the serum glycoprotein samples. This latter advantage is in addition to the following advantages: (1) a reduction in standard deviation, and (2) a reduction in the amount of interference produced by nonfucose moieties so that a fucose

value is obtained which more closely resembles the "true" fucose level than the manual procedure published by Winzler.[6]

The only apparent disadvantage to the automated method is the same as that of any other automated method employing concentrated sulfuric acid under constant pressure. Care must be taken to insure that all manifold tubing and connections are properly assembled and free of any restrictions.

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